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BIOTECHNOLOGY

# The Polyextremophilic Bacterium *Clostridium paradoxum* Attains Piezophilic Traits by Modulating Its Energy Metabolism and Cell Membrane Composition

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**ABSTRACT** In polyextremophiles, i.e., microorganisms growing preferentially under multiple extremes, synergistic effects may allow growth when application of the same extremes alone would not. High hydrostatic pressure (HP) is rarely considered in studies of polyextremophiles, and its role in potentially enhancing tolerance to other extremes remains unclear. Here, we investigated the HP-temperature response in *Clostridium paradoxum*, a haloalkaliphilic moderately thermophilic endospore-forming bacterium, in the range of 50 to 70°C and 0.1 to 30 MPa. At ambient pressure, growth limits were extended from the previously reported 63°C to 70°C, defining *C. paradoxum* as an actual thermophile. Concomitant application of high HP and temperature compared to standard conditions (i.e., ambient pressure and 50°C) remarkably enhanced growth, with an optimum growth rate observed at 22 MPa and 60°C. HP distinctively defined *C. paradoxum* physiology, as at 22 MPa biomass, production increased by 75% and the release of fermentation products per cell decreased by >50% compared to ambient pressure. This metabolic modulation was apparently linked to an energy-preserving mechanism triggered by HP, involving a shift toward pyruvate as the preferred energy and carbon source. High HPs decreased cell damage, as determined by Syto9 and propidium iodide staining, despite no organic solute being accumulated intracellularly. A distinct reduction in carbon chain length of phospholipid fatty acids (PLFAs) and an increase in the amount of branched-chain PLFAs occurred at high HP. Our results describe a multifaceted, cause-and-effect relationship between HP and cell metabolism, stressing the importance of applying HP to define the boundaries for life under polyextreme conditions.

**IMPORTANCE** Hydrostatic pressure (HP) is a fundamental parameter influencing biochemical reactions and cell physiology; however, it is less frequently applied than other factors, such as pH, temperature, and salinity, when studying polyextremophilic microorganisms. In particular, how HP affects microbial tolerance to other and multiple extremes remains unclear. Here, we show that under polyextreme conditions of high pH and temperature, *Clostridium paradoxum* demonstrates a moderately piezophilic nature as cultures grow to highest cell densities and most efficiently at a specific combination of temperature and HP. Our results highlight the importance of considering HP when exploring microbial physiology under extreme conditions and thus have implications for defining the limits for microbial life in nature and for optimizing industrial bioprocesses occurring under multiple extremes.

**KEYWORDS** PLFA, endospore, fermentation, halophiles, hydrostatic pressure, piezolyte, piezophiles, polyextremophiles, propidium iodide, thermophiles

The microbial world holds great metabolic flexibility which allows microorganisms to colonize even extreme environmental niches. By definition, microbes able to grow under one extreme condition are called extremophiles, while those able to grow under

**Citation** Scoma A, Garrido-Amador P, Nielsen SD, Røy H, Kjeldsen KU. 2019. The polyextremophilic bacterium *Clostridium paradoxum* attains piezophilic traits by modulating its energy metabolism and cell membrane composition. Appl Environ Microbiol 85:e00802-19. https://doi.org/10.1128/AEM.00802-19.

**Editor** M. Julia Pettinari, University of Buenos Aires



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**Received** 6 April 2019
**Accepted** 13 May 2019
**Accepted manuscript posted online** 24 May 2019
**Published** 18 July 2019

two or more extremes are called polyextremophiles (1). Of the numerous environmental factors with the potential to shape microbial activity, only the combination of a few of them (e.g., temperature, pH, and salinity) has been studied in detail (2). In particular, the impact on microbial physiology of hydrostatic pressure (HP) in combination with other such factors has been rather neglected; despite this, HP represents a distinctive factor in the deep sea, the largest reservoir of microbial diversity on Earth (3).

Piezophiles (pressure-loving) microbes are defined as those growing optimally at 40 MPa or higher (4). An increase in HP up to 50 MPa (equivalent to 5,000 m below seawater level [bsl]) may impair biological processes such as cell division, motility, and membrane protein function (5, 6). For instance, at high HP, the increased stabilization of hydrogen bonds in DNA may hinder DNA replication (7). Similarly, HP may change the conformation of multimeric complexes, such as ribosomes (8), and the structural context of membrane proteins thereby affecting their function and, more in general, cell homeostasis (reviewed in reference 9). Piezophilic prokaryotes adapted to HP are characterized by a high relative abundance of unsaturated phospholipid fatty acids (PLFAs) (in bacteria) (10) or glycerol ether lipids (archaea) (11), by the accumulation of organic solutes, the so-called piezolytes (12), and by specific proteome and genome responses, e.g., the expression of cold and heat shock proteins or alternative respiratory pathways (6, 13, 14). Some of these strategies are shared by prokaryotes exposed to other extremes, e.g., psychrophiles also show a high proportion of unsaturated PLFAs in their cell membranes, and halophiles and thermophiles accumulate organic solutes intracellularly (called osmolytes and thermolytes, respectively) (1). Whether exposure to additional extremes increases or decreases the capacity to tolerate high HP is poorly resolved, perhaps due to the few polyextremophilic isolates available in culture collections which are also known to be piezophilic (15).

In the present study, we addressed this question by testing the impact of combined HP-temperature variations on the growth and physiology of the endospore-forming moderately thermophilic anaerobic haloalkaliphile *Clostridium paradoxum*. The optimal growth conditions of *C. paradoxum* reportedly are pH 10.1, 56°C, and 50 to 200 mM NaCl (16), while its ability to grow at elevated HP was not tested thus far. We hypothesized that the capacity of *C. paradoxum* to grow at pH values up to 11.1, maximum temperatures of 63°C, and salinities up to 1 M NaCl (16) could enable its growth also at high HPs. In particular, the reduced cell membrane fluidity exerted by enhanced HP may be compensated by a higher growth temperature, which tends to increase membrane fluidity. Combined exposure to high HP and temperature may thus expand the growth limits of *C. paradoxum*, as previously reported for *Escherichia coli* (17) and a hyperthermophilic *Pyrococcus* strain (18). To test this hypothesis, we determined the growth rate, growth yield, piezolyte production, and membrane phospholipid composition of *C. paradoxum* cultures grown under 9 different combinations of temperature and HP.

## RESULTS

**Few combinations of elevated HP and temperature have positive effects on growth.** The growth of *C. paradoxum* with YTP medium (see Materials and Methods) was examined in a test matrix of 50 to 70°C and 0.1 (atmospheric pressure) to 30 MPa. At 50°C, application of high HP had a consistent negative effect on the cells' capacity to grow (Fig. 1A). During a 4-h incubation period, the net increase in cell number was reduced already at 15 MPa, and no net growth was observed at 30 MPa. On the contrary, at 60°C, a stimulation of culture growth was observed when increasing the HP from 0.1 to 15 MPa, although a further increase to 30 MPa resulted in a reduced, but not abolished, growth capacity. At 70°C, no substantial increase in cell density was observed at any elevated HP; however, weak but consistent growth occurred at 70°C at ambient pressure.

Generally, an increase in *C. paradoxum* cell numbers was consistent with a decrease in the pH of the culture medium, as expected for a culture growing by fermentation (Fig. 1B). At 70°C, a slight decrease in pH was observed also in noninoculated controls (data not shown). This decrease was comparable to the decrease in pH in inoculated

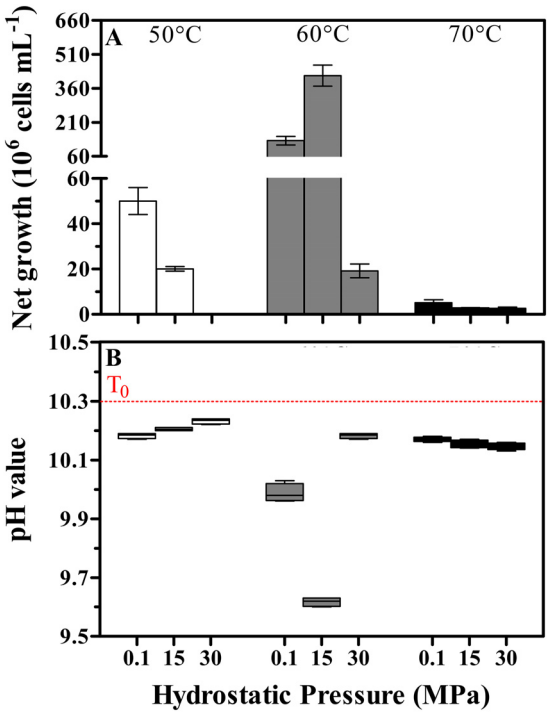


**FIG 1** (A and B) Net increase in cell numbers (A) and resulting decrease in pH (B) in cultures of the fermentative bacterium *C. paradoxum* grown for 4 h at different temperatures (50, 60, or 70°C) and hydrostatic pressures (0.1, 15, or 30 MPa). Hydrostatic pressure had a positive impact on growth only at 60°C. The results are the means from experiments made in three independent replicates. The dotted line at time zero ($T_0$) indicates the pH at the onset of the experiment.

cultures (Fig. 1B), thus explaining the acidification associated with weak growth at 70°C (Fig. 1). As *C. paradoxum* reportedly has a maximum growth temperature of 63°C (16), new tests were conducted to verify the observed growth at 70°C. In these tests, the inoculum was either increased from 1% to 20% (vol/vol) or derived from actively growing cultures (cultivated at 60°C and 15 MPa). These additional growth tests confirmed that in our cultivation system using YTP medium, *C. paradoxum* can in fact grow, albeit slowly, at 70°C and 0.1 MPa but not at 15 or 30 MPa. As such, a growth-promoting temperature-HP interaction only occurred at 60°C among the tested temperatures. Notably, stress conditions may lead to the formation of filamentous cells in *C. paradoxum* (e.g., high pH [16]), which may bias flow cytometry and optical density measurements. Therefore, cell morphology was assessed at the end of growth experiments by phase-contrast microscopy; however, no filamentous cells were observed.

***C. paradoxum* shows optimal growth at 22 MPa and 60°C.** The growth of *C. paradoxum* at 60°C was investigated further in a second experiment testing several HPs in the range of 0.1 to 30 MPa (Fig. S2). The growth rate was steadily enhanced by an HP increase from 0.1 to 22 MPa, with the application of 30 MPa still resulting in net growth but at a lower rate than with 22 MPa (Fig. S2A). The cell number remained almost unchanged during the first hour of incubation, with the positive impact of HP on cell division becoming evident already after 2 h. All cultures entered the exponential phase between 2 and 2.5 h of incubation (Fig. S2A), and growth rates were calculated using this time period. The fastest growth was observed at 60°C and 22 MPa, where the minimum generation time (11.0 $\pm$ 1.6 min) resulted in the maximum observed final cell number ($344 \times 10^6 \pm 7.2 \times 10^6$ cells $ml^{-1}$ within 4 h of incubation (Fig. 2). The inoculation strategy adopted in this experimental set (i.e., using a higher relative abundance of vegetative cells) increased the final number of cells at 0.1 and 30 MPa; however, it reduced the final cell number observed at 15 MPa (compare Fig. 1A and 2). Phase-contrast microscopy examination of cultures grown for 4 h at 15 MPa revealed that 34.2%

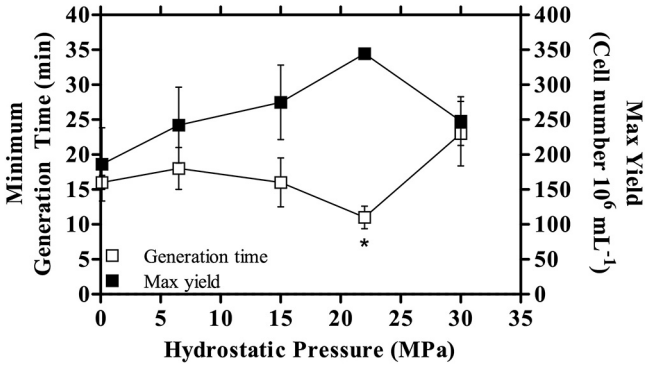


**FIG 2** Minimum generation time and maximum (Max) cell number observed in cultures of *C. paradoxum* upon 4 h of cultivation at 60°C at different hydrostatic pressures (0.1, 6.5, 15, 22, or 30 MPa). The highest cell number and shortest generation time occurred at 22 MPa. Growth rates were calculated considering the increase in cell number between 2 and 2.5 h of incubation, that is, when cells grew exponentially at any tested hydrostatic pressure. Cultures grown at 0.1 and 22 MPa reached their maximum cell number in 3.5 h, contrary to all others, which required 4 h. *, the generation time observed at 22 MPa was statistically different from all other data points, according to a 95% confidence interval (95% CI) calculated using a Student test with a two-sided distribution. Statistical significance was assessed using a nonparametric test (Mann-Whitney test), which considered a two-sided distribution with 95% CI. The results are the means from experiments made in five independent replicates.

(±3.3%) of the cells were already sporulating, likely as a result of high growth rates. On the contrary, cultures grown at 0.1 and 30 MPa were almost exclusively composed of vegetative cells (see Table S1 in the supplemental material), indicating that under these culture conditions, the sporulation process was not directly influenced by HP.

Through the 4-h incubation period, the number of damaged cells increased at any of the tested HPs, as assessed by staining with Syto9 and propidium iodide using flow cytometry (Fig. 3A). However, the relative abundance of damaged cells was reduced from 90% to 45% of the total number of cells when HP was increased from 0.1 to 30 MPa, indicating that cell integrity remarkably benefited from an HP increase. For the convenience of comparison, data from Fig. S2A are also reported in Fig. 3B; this highlights the positive impact of high HP, which resulted in cultures with proportionally fewer damaged cells (Fig. 3A) and higher cell densities (Fig. 3B). Provided that the release of endospores may leave cytoplasmic membranes of mother cells damaged, a possible correlation between cell damage and sporulation as a function of HP was investigated; however, no correlation was found (Table S1). Taken together, the enhanced growth rates, biomass yields, and cell integrity observed at elevated HP (Fig. 1 and 3) are suggestive of a piezophilic nature of *C. paradoxum*. The possibility that high

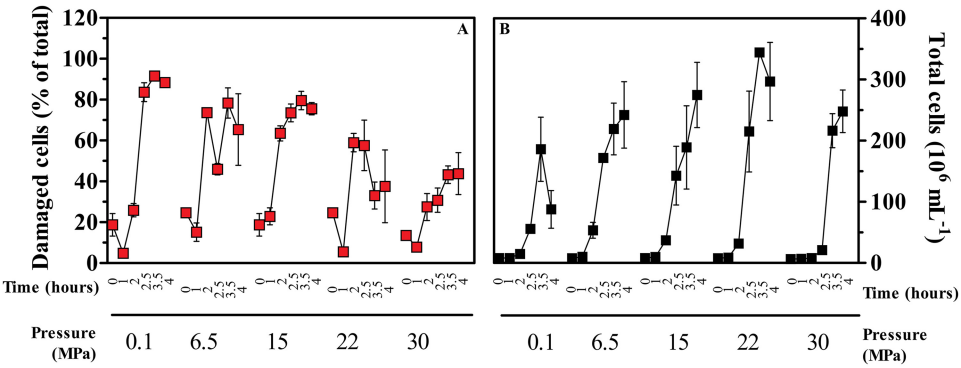


**FIG 3** (A and B) Time course of damaged (A) and total cell counts (B) during growth of *C. paradoxum* cultures at 60°C under different hydrostatic pressures (0.1, 6.5, 15, 22, or 30 MPa). Damaged cells were assessed by LIVE/DEAD staining using both Syto9 and propidium iodide, while total cell counts were obtained by staining only with Syto9. Data from Fig. S2A are used in panel B for convenience of comparison. The results are the means from experiments made in five independent replicates.

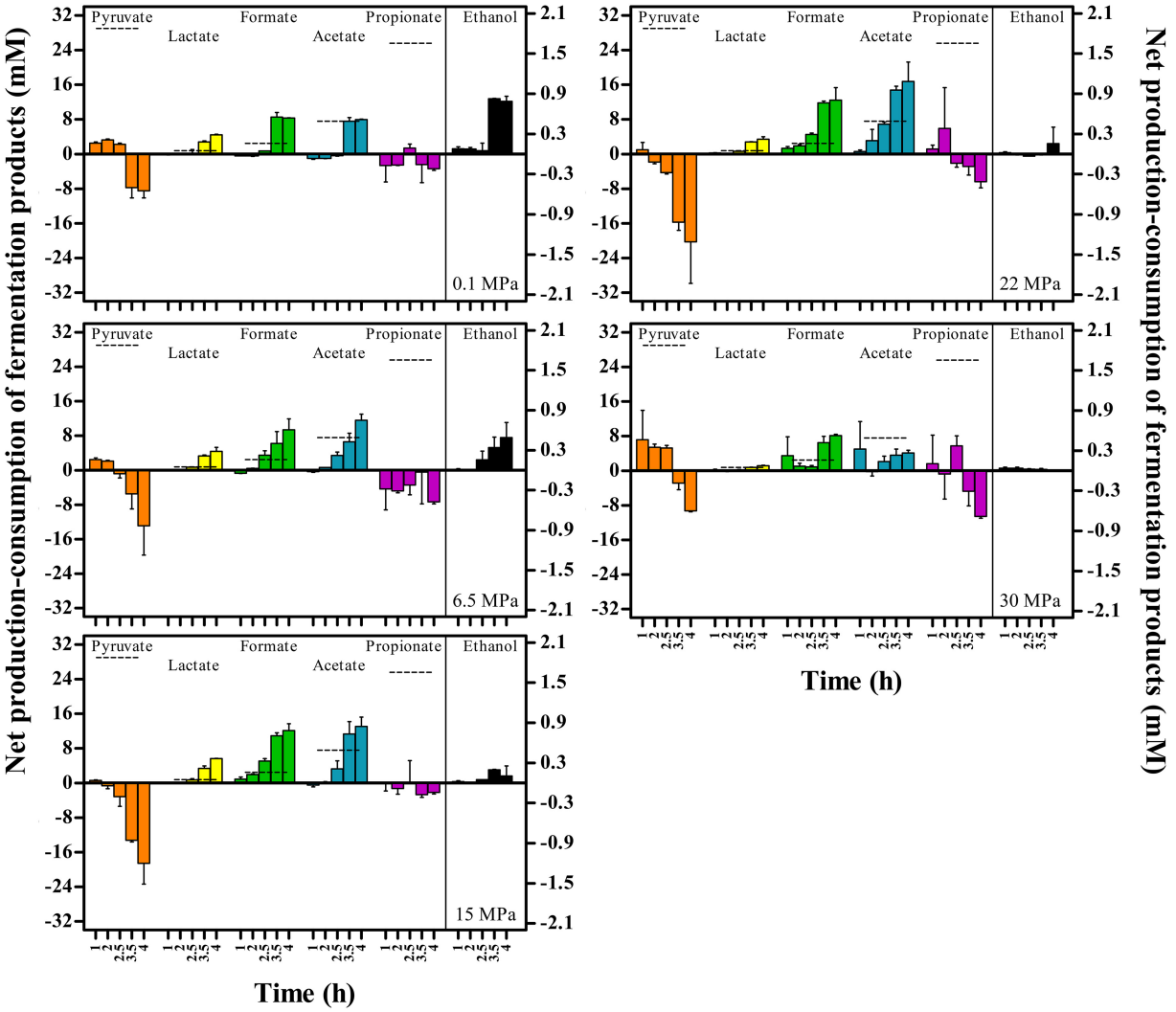


**FIG 4** Net production and consumption of fermentation products in *C. paradoxum* cultures grown at 60°C at different hydrostatic pressures (0.1, 6.5, 15, 22, or 30 MPa). Ethanol refers to the left *y* axis. Dotted lines above each set of bars indicate the initial concentration of each compound in the cultivation YTP medium. Ethanol has no dotted line, as it is absent in YTP medium. These data refer to the growth curves shown in Fig. 2 and 3. The results are the means from experiments made in five independent replicates.

growth rates or enhanced cell integrity was linked to the accumulation of intracellular solutes was tested; however, no potential piezolyte was detected under the optimal growing conditions of 22 MPa and 60°C (Fig. S3).

**Increased HP changes fermentation patterns and increases growth yield.** When grown on YTP medium, *C. paradoxum* formed lactate, formate, acetate, and ethanol as the major fermentative end products (Fig. 4). The incubation experiments were performed in vials filled to capacity with culture medium, which allowed testing of cultures under increased HP. As a result, microbially produced $CO_2$ only accumulated in the liquid phase; however, gas chromatography measurements of dissolved inorganic carbon ($CO_2 + H_2CO_3 + HCO_3^- + CO_3^{2-}$) showed no consistent accumulation above the background level of the YTP medium (data not shown). As in the absence of a gas phase, $H_2$ gas could hardly accumulate in the liquid phase (at 60°C, $H_2$ gas solubility in water is saturated below 1.2 mg $kg^{-1}$ [https://www.engineeringtoolbox.com/]), its role as a fermentation product in our experiments was of minor importance. Net formation of pyruvate and propionate was detected throughout the experiment (Fig. 4). In particular, pyruvate slightly accumulated during the first hours of incubation, indicating that *C. paradoxum* used other carbon sources present in the YTP medium (Fig. 4), such

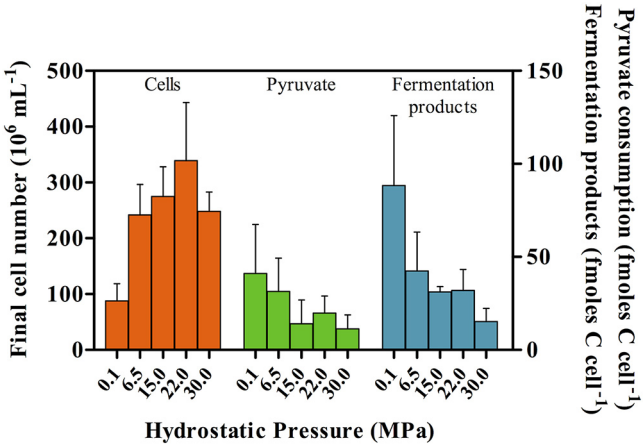


**FIG 5** Final cell number, pyruvate consumption, and total fermentation products generated in *C. paradoxum* cultures grown for 4 h at 60°C at different hydrostatic pressures (0.1, 6.5, 15, 22, or 30 MPa). Pyruvate consumption and fermentation products generated were normalized to cell numbers to highlight hydrostatic pressure impact on cell metabolism. The data refer to the end of the incubation period (4 h) when pyruvate consumption was observed under all hydrostatic pressure conditions. The final cell number from Fig. 2 is shown for convenience of comparison. The results are the means from experiments made in five independent replicates. Error bars indicate standard deviations.

as amino acids, which may lead to the generation of pyruvate through Stickland reactions (19). Notably, Stickland reactions circumvent $H_2$ accumulation by using amino acids as both an electron donor and acceptor. Nonetheless, pyruvate was always consumed at the end of the 4-h incubation period (Fig. 4). Its consumption was faster and more pronounced at increasing HP (compare the time courses for pyruvate production/consumption at different HPs, Fig. 4), indicating that pyruvate became the preferential carbon source earlier during the growth of the cultures. Increasing HP also impacted the net consumption-production profile of other fermentation products: for example, ethanol concentration decreased with increasing HP, and it was almost undetectable at 30 MPa (detection limit, 0.03 mM; Fig. 4). A more accurate insight into HP impact on cell metabolism was assessed by calculating pyruvate consumption and fermentation product generation on a per-cell basis (Fig. 5). Although the final cell numbers increased with increasing HPs (up to 22 MPa), cellular pyruvate consumption and fermentation product formation decreased (Fig. 5). This suggests that the growth yield increased at elevated HP. In this respect, it must be noted that at 0.1 MPa, the amount of carbon released as fermentation products was greater than the amount of carbon derived from pyruvate consumption (Fig. 5), which shows that alternative carbon sources in the YTP medium were catabolized at ambient pressure. However, in cultures grown at 22 and 30 MPa, this ratio is more stoichiometrically balanced (Fig. 5), further supporting the hypothesis that pyruvate became the main catabolic substrate at high HPs.

**High HP increases branched-chain PLFAs and reduces PLFA chain length.** In agreement with previous observations (16), *C. paradoxum* cells contained PLFAs with 13 to 18 carbon atoms, with the large majority between 15 and 17, irrespective of the cultivation conditions (Fig. S4). HP distinctively shaped PLFA profiles as total branched-chain fatty acids increased with HP (relative abundance up to 80% of all PLFAs), with an abrupt increase already observed from 0.1 to 6.5 MPa (Fig. 6). Only negligible effects of HP on the proportion of saturated PLFAs were observed, as >95% were saturated under all cultivation conditions (Fig. 6). Another prominent effect of HP on the membrane PLFA composition was a reduction in PLFA chain length with increasing HP. This shortening occurred in all major types of PLFAs, as evidenced by the ratios between short and long PLFAs (e.g., saturated, $C_{14:0}$ to $C_{16:0}$; and branched chain, $C_{15:0}$ to $C_{17:0}$ and $C_{13:0}$ to $C_{17:0}$) (Fig. 6).

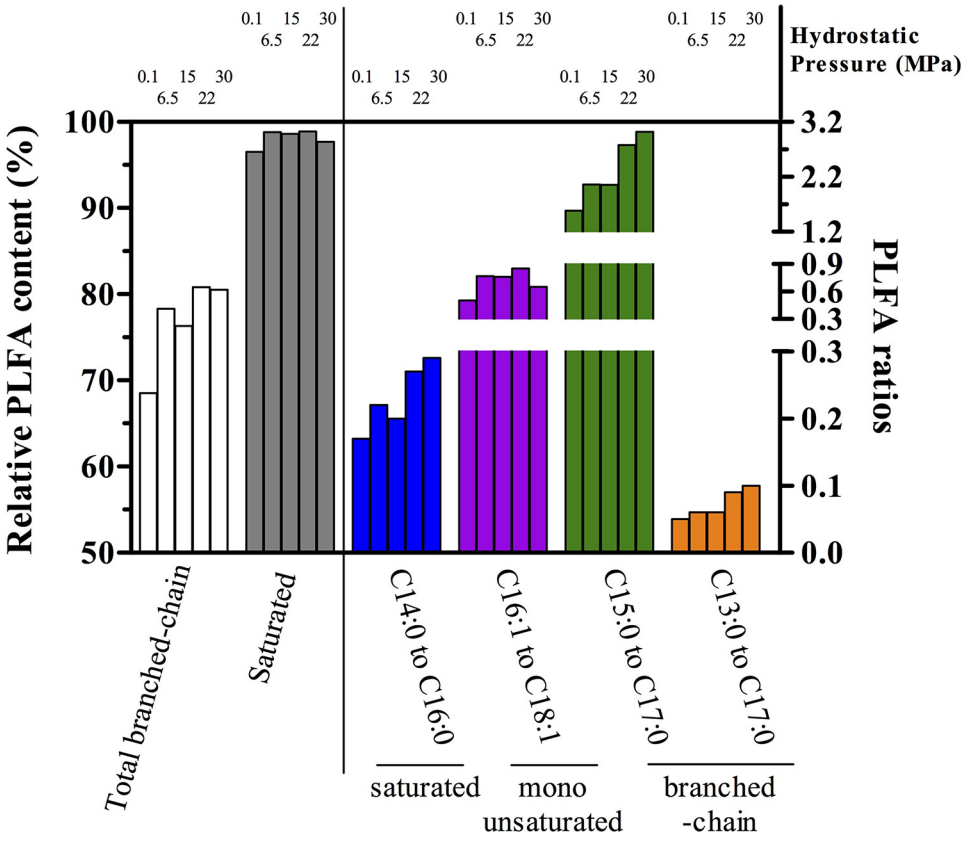


**FIG 6** Changes in the relative abundances of selected membrane phospholipid fatty acids (PLFAs) in *C. paradoxum* cultures grown at 60°C at different hydrostatic pressures (0.1, 6.5, 15, 22, or 30 MPa). PLFAs were grouped according to their biochemical composition. Cultures from three independent vials of 15 ml each were pooled to generate one pellet per hydrostatic pressure to have enough material for the analysis.

## DISCUSSION

*C. paradoxum* showed the highest growth rates when cultivated at 22 MPa (equivalent to the pressure experienced about 2,200 m bsl), 60°C, and initial pH 10 (Fig. 2). The moderately piezophilic nature of this anaerobe, along with its halotolerance and thermo- and alkaliphilicity, is most surprising provided it has been repeatedly retrieved from sewage treatment plants in the United States, where alternating oxygen availability occurred along with sodium concentrations of <3.5 mM, pH < 7.6, and temperatures of <38°C (16). Cultivable prokaryotic isolates with a demonstrated thermo- and piezophilic physiology typically originate from deep-sea hydrothermal vent systems (20). Many such isolates belong to the domain *Archaea* and only a few belong to *Bacteria*, e.g., *Marinitoga piezophila* (21), *Thermosipho japonicus* (22), *Thioprofundum lithotrophica*, *Piezobacter thermophilus* (23), and *Desulfovibrio hydrothermalis* (24). However, these bacterial isolates have pH optima between 6.0 and 7.8, far below the pH optimum of *C. paradoxum*. The tolerance of *C. paradoxum* to multiple extremes and its fast growth (generation time, 11 min) make it a candidate model organism to study the physiology and adaptations of polyextremophilic bacteria. Of the 66 isolated strains with demonstrated polyextremophilicity, only 17 are known to grow more at increased HP (i.e., 25%, as recently estimated [15]). Cultivation of the remaining polyextremophilic isolates may reveal that many more are actually stimulated by high HP. This consideration may extend to nonpolyextremophilic microbes, as extensive evidence suggests that HP represents a key parameter influencing biochemical reactions and microbial physiology to the same extent as more commonly studied physicochemical factors, e.g., pH, temperature, and salinity (25).

Three main physiological traits were characteristic of *C. paradoxum* growth under

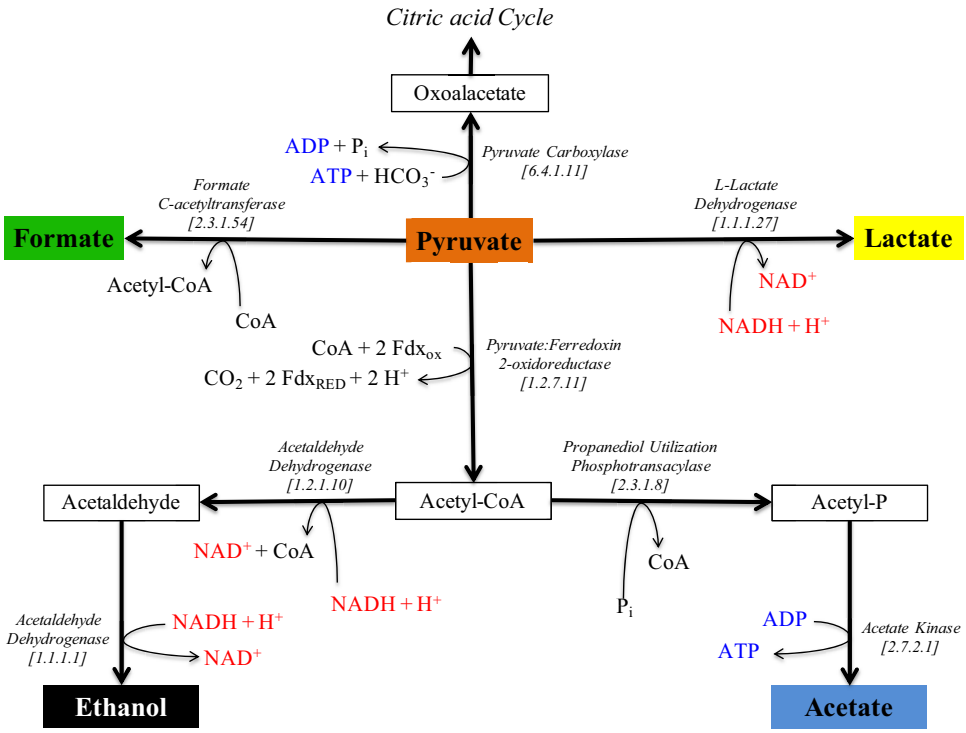


**FIG 7** Proposed metabolic pathways and enzyme EC numbers indicating the pathways of fermentative metabolism of pyruvate in *C. paradoxum*. From pyruvate, ATP is formed when producing acetate and formate (via acetyl-CoA). All the enzymes shown are encoded in the genome of *C. paradoxum* DSM 7308 with the following locus tags (IMG project ID Gp0102900): pyruvate carboxylase, Ga0056075_00825; formate C acetyltransferase, Ga0056075_00436; L-lactate dehydrogenase, Ga0056075_00208 (putative annotation); pyruvate_ferredoxin 2-oxidoreductase, Ga0056075_01750; acetaldehyde dehydrogenase, Ga0056075_01139; acetaldehyde dehydrogenase, Ga0056075_01139; propanediol utilization phosphotransacylase, Ga0056075_01752; and acetate kinase, Ga0056075_01188. *, putative annotation.

polyextreme conditions of high HP (up to 30 MPa), temperature (60°C), and pH (initially 10), as follows: (i) an increased growth rate and yield (Fig. 2 and S2), (ii) the lack of accumulation of intracellular organic solutes (Fig. S4), and (iii) a modified profile of membrane PLFAs (Fig. 6).

The increased growth was apparently associated with the modulation of fermentative metabolism. A main substrate in the growth medium was pyruvate, whose fermentation to acetate and formate yields ATP via the formation of acetyl-coenzyme A (acetyl-CoA) (Fig. 7). The ratio between the consumption of pyruvate and formation of acetate and formate remained fairly constant in growth experiments from 0.1 to 22 MPa (Fig. S5), when a positive correlation between HP and biomass yield was observed (Fig. 2). On the contrary, the formation of lactate and ethanol per pyruvate consumed decreased with increasing HP (Fig. S5). The formation of lactate and ethanol from pyruvate does not yield ATP but results in the regeneration of oxidized electron carriers (i.e., $NAD^+$, Fig. 7). Their production is therefore likely linked to the fermentation of substrates other than pyruvate. Thus, such substrates would be consumed less at increasing HP. In agreement with this, the carbon budget of pyruvate consumption and fermentation product formation became more balanced at elevated HP (Fig. 5).

The shift toward the use of pyruvate as the preferred growth substrate and increased growth at elevated HP was accompanied by a decrease in pyruvate consumption per cell (Fig. 5), suggesting that *C. paradoxum* responded to HP by increasing growth yield (i.e., increased amount of biomass produced per resource consumed [26, 27]). Improved growth yields are commonly attained by selectively associating the uptake of growth substrates to central metabolism and its related assimilatory pathways (28). *C. paradoxum* may thus "invest" in pyruvate uptake as a means to optimize energy generation at high HP. Other piezophilic bacteria (e.g.,

*Desulfovibrio hydrothermalis* and *Desulfovibrio piezophilus*) also attain high growth yields at increased HP through energy conservation strategies (29). An HP-selective inhibition of the enzymes involved in the fermentation pathways leading to ethanol and lactate production (Fig. 7) cannot be dismissed to explain the observed results (Fig. 5 and S5); however, this hypothesis appears unlikely. First of all, this random inhibition would then fortuitously lead to increased growth (Fig. 2); second, lactate dehydrogenase generally shows low sensitivity to high HP (30, 31).

Variations in temperature, salinity, HP, or pH in the external environment may affect cellular turgor pressure by impacting the water influx to the cell. In the case of strong alterations between cell cytoplasm and external environment, the cell wall and membrane may be damaged (32). In *C. paradoxum*, increased HP reduced the number of damaged cells (Fig. 3), indicating either that atmospheric pressure exerted a membrane stress or that elevated HP somehow preserved membrane integrity. A higher relative abundance of damaged cells is normally observed when exposing cultures to elevated HP (33), with bacteria sometimes able to accumulate intracellular organic solutes to counterbalance the effects of increased HP. The observation that certain solutes linearly accumulate in deep-sea fish at increasing water depths (reviewed in reference 34) led to the hypothesis that such solutes (termed "piezolytes" [12]) would directly or indirectly counteract increases in HP independent of other factors (i.e., salinity and temperature). While the list of marine organisms and microorganisms accumulating intracellular solutes at high HP has been increasing over the years (12, 29, 35–41), the potential role of piezolytes in conferring HP tolerance remains fairly unclear. Microbial piezolyte producers were either moderate piezophiles ($\beta$-hydroxybutyrate in Photobacterium profundum [12] and glutamate in *D. piezophilus* [29]) or piezosensitive (ectoine in *Alcanivorax borkumensis* [39, 41] and *N*-trimethylamine oxide [TMAO] in *Vibrio fluvialis* [40]). In the moderate piezophile *C. paradoxum,* no organic solute accumulated intracellularly under optimal growth conditions (i.e., 22 MPa, 60°C, pH 10, 90 mM NaCl; Fig. S4). Similarly, in the hyperthermophilic piezophilic archaeon Thermococcus barophilus, accumulation of the osmolyte mannosyl-glycerate was observed at low suboptimal HPs rather than at the optimal HP of 40 MPa (42). The *de novo* synthesis of organic solutes is expected to be energetically costly and at the expense of energy available for cell division (43). The accumulation of piezolytes would thus appear a counterintuitive strategy considering the hypothesized energy-preserving mechanisms triggered by HP in *C. paradoxum*. Accumulation of organic solutes is common in thermophiles which are also halotolerant (44), such as *C. paradoxum*, which can tolerate up 1 M NaCl, in the presence of 25 mM KCl, at 50°C, pH 9.6, and 0.1 MPa (16). The genome of *C. paradoxum* encodes glycine betaine transporter family proteins (locus tags Ga0132923_111649 and Ga0056075_01584, which are homologous to the EctT transporter protein in *Virgibacillus pantothenticus* involved in ectoine uptake [45]) and a trehalose-specific phosphotransferase (PTS) sugar transporter system (locus tags Ga0056075_01596 and Ga0132923_111867). Glycine betaine and trehalose are known organic solutes (34); however, their potential as piezolytes has never been reported. Their possible role in supporting *C. paradoxum* growth at elevated HP remains to be investigated; however, our present data set suggest that *C. paradoxum* does not rely on piezolytes to attain its piezophilic traits.

An increase in HP affects fatty acids in biological membranes by increasing their packing and changing their conformation, reducing the membrane's capacity to regulate proton and water influx/efflux (9, 46). Cells may respond to changes in HP by adapting the biochemical composition of the fatty acids in their membrane to maintain its functional viscosity ("homeoviscous adaptation" [47]) or to prevent lipids from forming nonbilayer structures which would disrupt membrane permeability ("homeophasic adaptation" [48]). Bacteria modify their fatty acids by changing the fatty acyl or alkyl composition, e.g., by changing their level of saturation, chain length, branching, and cyclization (49). In *C. paradoxum*, high HP resulted in an increase in branched-chain PLFAs, along with a general shortening of PLFA chain length (Fig. 6). Normally, an increase in HP results in a higher degree of mono- or polyunsaturated PLFAs in both

piezophilic and piezosensitive bacteria (50–56). In contrast to most studies (conducted on deep-sea bacteria living at about 5°C and neutral pH), *C. paradoxum* was cultivated at high temperature and pH (60°C, pH 10). High temperature has the opposite effect of high HP, as it decreases the viscosity of cell membranes (51). Cells respond to high temperature by typically synthesizing longer (57, 58) and more saturated (59) PLFAs. By penetrating further into the phospholipid bilayer, longer chains increase acyl chain interactions between layers, conferring rigidity to cell membranes (60). However, fatty acid saturation has a much stronger effect on cell membrane rigidity than does chain length (49). In fact, at 60°C *C. paradoxum* had more than 90% saturated PLFAs irrespective of the HP applied (Fig. 6). We speculate that modulating the membrane composition to consist of more saturated branched-chain and shorter PLFAs may be one of few viable strategies to cope with the concomitant increase of temperature and HP. As short PLFA chains are less restricted by chain interforces, they result in more fluid structures (60). Branching of fatty acid chains lowers the melting point temperature of membranes compared to equivalent straight-chain fatty acids (46), and it does not support the formation of crystalline structures between adjacent acyl chains (61), thereby increasing membrane fluidity. By increasing the relative abundance of short- and branched-chain membrane PLFAs from 0.1 to 30 MPa (Fig. 6), *C. paradoxum* would thus enhance its membrane fluidity, a desirable trait at high HP. Accumulation of branched-chain PLFAs is not an unusual response to high HPs, as it was observed in a Gram-positive piezotolerant bacterium grown at $>$30 MPa and 35°C (62). However, branched-chain PLFAs also retain a functional bilayer that is still not too fluid at high temperatures (63), a critical feature for *C. paradoxum* cells growing at 60°C. In fact, branched-chain PLFAs are hallmarks of thermophilic bacteria (64). Strategies to maintain membrane fluidity differ among bacteria; however, unsaturation of PLFAs may be essential only in bacteria with straight-chain PLFAs (65).

The homeoviscous/homeophasic adaptation is a general mechanism responsive to different environmental factors (20). By acting on protons and water influx/efflux, variations in salinity or pH may be perceived by the cell in a manner similar to that of an HP or temperature shift. This has been reported in *C. paradoxum* when exposed to "stressing" neutral pH values (i.e., 7.5) at atmospheric pressure, which led to an identical (if not stronger) reduction of PLFA chain length as with high HP (Fig. S6) (16). Notably, the relative abundance of total branched-chain PLFAs remained unchanged following such a pH shock, confirming that their accumulation in *C. paradoxum* represents a specific response to elevated HP at high temperature (Fig. S6).

Cultivation is a direct means to establish the cause-effect relationship between environmental parameters and microbial metabolism. This is particularly true for polyextremophilic bacteria, with HP being a widely neglected factor thus far. By systematically investigating the temperature-HP response of the polyextremophile *C. paradoxum,* we found that its maximum temperature is 7°C higher than what was previously reported (now 70°C) and that its highest growth rate and growth yield occur at 60°C and pH 10 when applying an HP of 22 MPa. This makes *C. paradoxum* a thermophilic alkaliphilic halotolerant moderately piezophilic anaerobic bacterium and, as such, a candidate model organism for studying microbial polyextremophily. In particular, the moderate piezophilic nature observed under polyextreme conditions of high pH and temperature was expressed as a result of (i) an energy-preserving mechanism shaping fermentation pathways, (ii) no piezo- or thermolyte accumulation despite an increased cell integrity, and (iii) an increase in the content of branched-chain PLFAs in its cell membrane, along with a reduction in PLFA chain length. Studies on *C. paradoxum* and other model polyextremophiles are particularly relevant to understand how microorganisms adapt to multiple extremes that would normally exert opposite cellular effects when applied independently, e.g., high temperature and HP. Such studies may ultimately help understand and define the physicochemical limits for microbial life.

## MATERIALS AND METHODS

**Strain and culture conditions.** All experiments were conducted using pure cultures of *C. paradoxum* strain JW-YL-7, which is the type strain of the species. Inocula were originally grown statically, under anoxic conditions in sealed vials of 8 ml, with a 6-ml working volume at 50°C under an atmosphere of 100% $N_2$. All cultures were grown in YTP medium (DSM medium 616), which was modified by substituting glucose with sodium pyruvate (3 g liter$^{-1}$). This substitution was necessary because of glucose "caramelization" in the medium at temperatures of >55°C. The final pH of the medium was adjusted to 10.0. All chemicals for medium preparation were from Sigma-Aldrich (Denmark).

All subsequent growth tests were conducted statically in tightly sealed 4-ml glass vials (TA Instruments, Denmark) with no gas phase. Sterile autoclaved vials were flushed with sterile $N_2$ for at least 1 min to remove oxygen. Then, 0.04 ml inoculum (i.e., 1%) and anoxic culture medium (3.96 ml) were injected serially by sterile $N_2$-flushed syringes to fill the vials to capacity. The inoculated vials were then placed in 200-ml high-pressure reactors (Classic Filters Ltd., UK), which can work in a pressure range of 0.1 to 40 MPa. After HP was increased manually through a pump (Enerpac, The Netherlands), pressure reactors were placed in a custom-made incubator where the temperature could be set in a range between 20 and 100°C by means of a water thermostat (Corio C; Julabo GmbH, Germany). The compression and decompression rate was equal to 120 MPa min$^{-1}$ (66), which is a conservative rate compared to the more commonly used 200 MPa min$^{-1}$ (67–69). All incubations lasted 4 h, after which a decrease in optical density was generally observed under the standard conditions of 50°C, 0.1 MPa, and pH 10. Experiments were made using three to five independent replicates.

**Growth experiments.** The growth response to temperature and HP was initially tested at 50, 60, and 70°C, with HPs of 0.1 MPa (atmospheric pressure), 15 MPa, and 30 MPa. Inocula were prepared as follows: after growth at 50°C, 0.1 MPa, and pH 10, cultures were stored at room temperature (20 to 25°C) for 2 to 3 weeks and inoculated as described above at an initial concentration of $19.5 \times 10^6 \pm 5.3 \times 10^6$ cells ml$^{-1}$, as assessed by flow cytometry (see below). These cultures were composed of 43.6% ± 2.0% vegetative cells, 23.8% ± 1.5% sporulating cells, and 32.5% ± 2.3% endospores (± standard deviation [SD], $n = 3$), as assessed by phase-contrast microscopy.

In a second set of experiments, growth was tested at 60°C, and HP was set at 0.1, 6.5, 15, 22, and 30 MPa. Experiments were initiated by inoculation with overnight-grown cultures, as this was observed to slightly reduce the duration of the initial lag phase, while having no effect on growth rate or maximum cell density under standard conditions (50°C, pH 10, and 0.1 MPa; Fig. S1). Inocula for the second experiment were prepared as follows: cell cultures were cultivated overnight (13 to 17 h) at 50°C, 0.1 MPa, and pH 10. The resulting cultures were used as an inoculum only one time during the following morning for a 4-h experiment at different HPs. Such inocula were composed of 63.3% ± 4.5% vegetative cells, 35.7% ± 3.6% sporulating cells, and 1.0% ± 1.2% endospores (±SD, $n = 3$) (as assessed by phase-contrast microscopy) and had an initial density of $7.7 \times 10^6 \pm 0.6 \times 10^6$ cells ml$^{-1}$ (as assessed by flow cytometry).

**Microbiological analysis.** The pH was measured with an InLab Micro pH electrode connected to a SevenCompact pH meter (Mettler Toledo, Denmark). The optical density was measured at 600 nm in sealed glass vials with a spectrophotometer (UVmini-1240; Shimadzu, Japan). Cell numbers were assessed by flow cytometry (NovoCyte analyzer; ACEA, Bioscience, USA), using NovoFlow (ACEA) as a sheath fluid, following established procedures (70). Cell cultures were diluted $10^3$- to $10^4$-fold in phosphate-buffered saline (PBS; 130 mM NaCl, 10 mM $NaP_i$ [pH 7.4]) and stained with either Syto9 (for total cells counts) or Syto9 and propidium iodide (for intact-damaged cell count, LIVE/DEAD BacLight bacterial viability kit; Invitrogen, Fisher Thermo Scientific, Denmark), using the manufacturer's instructions. The LIVE/DEAD staining was performed, as it is used in high-HP treatments to study the capacity of cultures to recover from cell membrane damage after being subjected to sublethal-HP shocks (71, 72).

The abundances of vegetative cells, sporulating cells, and endospores in *C. paradoxum* cultures were determined from cell counts in a Bürker-Türk chamber by phase-contrast microscopy.

**Chemical analysis.** Fermentation products were analyzed on a high-performance liquid chromatography (HPLC) instrument (Ultimate 3000; Thermo Scientific, Denmark), using a Kinetex column (5 $\mu$m, $C_{18}$, 100 Å; Phenomenex, USA), an eluent phase of 5 M $H_2SO_4$ flowing at 0.6 ml min$^{-1}$, and a column temperature of 40°C. PLFAs were analyzed from pelleted cells after growth at different HPs at 60°C. For each HP tested, cultures from three independent vials of 15 ml each were pooled and pelleted by centrifugation to obtain enough material for PLFA analysis. Pellets were freeze-dried overnight (Micro-Modulyo; Edwards High Vacuum, UK) and subsequently sent to Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ; Braunschweig, Germany) for analysis. Intracellular organic solutes were analyzed on freeze-dried bacterial pellets by nuclear magnetic resonance (NMR) spectroscopy. First, intracellular organic solutes were extracted from freeze-dried bacterial pellets with ethanol, as previously described (73). Extracts were then freeze-dried, dissolved in water, and cleansed of lipid components through chloroform extraction. The resulting aqueous extracts were freeze-dried and resuspended in $D_2O$ for NMR analysis. NMR spectra were acquired on an Avance III 500 spectrometer (Bruker, Rheinstetten, Germany) working at a proton operating frequency of 500.13 MHz equipped with a CryoProbe Prodigy triple resonance probe (TCI). $^1$H-NMR spectra were acquired with water presaturation.

**Genome analysis.** The genomes of *C. paradoxum* DSM7308 and JW-YL-7$^T$ were analyzed for the presence of genes involved in the uptake or biosynthesis of organic solutes with the potential to act as piezolytes, using the IMG platform (74), where the genome sequences are available under IMG genome identifiers (IDs) 2599185174 and 2690315891.

**Statistical analysis.** The results were expressed as mean values from independent replicates ($n = 3$ or 5). Error bars in graphs indicate a 95% confidence interval (95% CI) calculated using a Student *t* test

with a two-sided distribution except for data in Fig. 5. Statistical significance was assessed using a nonparametric test (Mann-Whitney test) which considered a two-sided distribution with 95% CI.

## SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AEM.00802-19.

**SUPPLEMENTAL FILE 1**, PDF file, 0.9 MB.

## ACKNOWLEDGMENTS

This work was funded by The Danish Hydrocarbon Research and Technology Center (DHRTC) through the Self-Healing Cement project.

Helena Santos and Pedro Lamosa (Universidade Nove de Lisboa, Portugal) are gratefully acknowledged for the NMR analysis on intracellular organic solutes. The NMR data were acquired at CERMAX, ITQB-NOVA, Oeiras, Portugal. Flow cytometry was performed at the FACS Core Facility, Aarhus University, Denmark. Alyssa Findlay is gratefully acknowledged for her assistance with high-performance liquid chromatography (HPLC) measurements. Lykke Beinta Bjærge Bamdali, Katrine Bay Jensen, and Anne Stentebjerg are acknowledged for their technical assistance.

A.S., H.R., and K.U.K. conceived and designed the experiments. A.S., P.G.-A., and S.D.N. performed the experiments. A.S. analyzed the data. A.S. and K.U.K. wrote the manuscript with input from all coauthors.

We declare no conflicts of interest.

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